Depletion of CCR5-Expressing Cells with Bispecific Antibodies and Chemokine Toxins: A New Strategy in the Treatment of Chronic Inflammatory Diseases and HIV

Hilke Brühl,* Josef Cihak,† Manfred Stangassinger,† Detlef Schlöndorff,* and Matthias Mack[1]*

The chemokine receptor CCR5 is expressed on the majority of T cells and monocytes in the inflammatory infiltrate of diseases such as rheumatoid arthritis, renal diseases, and multiple sclerosis. In contrast, little expression of CCR5 is found on peripheral blood leukocytes. A specific depletion of CCR5$^+$ cells could therefore be a useful strategy to reduce the cellular infiltrate in chronic inflammations. Moreover, CCR5 is the major coreceptor for M-tropic HIV-1 strains. Depletion of CCR5$^+$ leukocytes may help to eliminate cells latently infected with HIV-1. We designed two constructs that specifically destroy chemokine receptor-positive cells. The first construct, a bispecific Ab, binds simultaneously to CCR5 and CD3. Thereby it redirects CD3$^+$ T cells against CCR5$^+$ target cells. The Ab specifically depletes CCR5$^+$ T cells and monocytes, but is inactive against cells that do not express CCR5. Furthermore, ex vivo the bispecific Ab eliminated >95% of CCR5$^+$ monocytes and T cells from the synovial fluid of patients with arthritis. Also, we designed a fusion protein of the chemokine RANTES and a truncated version of *Pseudomonas* exotoxin A. The fusion protein binds to CCR5 and down-modulates the receptor from the cell surface. The chemokine toxin completely destroyed CCR5$^+$ Chinese hamster ovary cells at a concentration of 10 nM, whereas no cytotoxic effect was detectable against CCR5$^-$ Chinese hamster ovary cells. Both constructs efficiently deplete CCR5-positive cells, appear as useful agents in the treatment of chronic inflammatory diseases, and may help to eradicate HIV-1 by increasing the turnover of latently infected cells. *The Journal of Immunology,* **2001, 166: 2420–2426.**

The chemokine receptor CCR5 is a member of a large family of G protein-coupled seven-transmembrane domain receptors that binds the proinflammatory chemokines RANTES, macrophage-inflammatory protein 1α (1), macrophage-inflammatory protein 1β, and monocyte chemotactic protein 2 (1, 2). Chemokines act in concert with adhesion molecules to induce the extravasation of leukocytes and to direct their migration to sites of tissue injury (3). In a variety of chronic inflammatory diseases, an impressive accumulation of CCR5-positive T cells and macrophages is found at the site of inflammation. An accumulation of CCR5$^+$ cells has been demonstrated in several types of arthritis (4, 5), inflammatory renal diseases including transplant rejection (6, 7), multiple sclerosis (8, 9), and inflammatory bowel diseases (10). In contrast, in the peripheral blood of these patients only a minority of T cells and monocytes express CCR5. Therefore, CCR5 appears to be an excellent marker to identify leukocytes that are involved in chronic inflammation. The occurrence of a 32-bp deletion in the CCR5 gene that prevents expression of CCR5 (11) allows study of the pathophysiological role of CCR5 in chronic inflammatory diseases. In patients with rheumatoid arthritis, the frequency of CCR5-deficient (CCR5-Δ32/Δ32)[2] (5) individuals is significantly reduced (12). Moreover, the mean survival of kidney transplants is significantly longer in CCR5-Δ32/Δ32 patients (D. Schlöndorff, personal communication). These results make CCR5 look like a promising target for therapeutic intervention. Furthermore, the predominance of CCR5-positive leukocytes in the diseased tissue in contrast to its rare expression on peripheral blood leukocytes suggests that a specific elimination of CCR5-positive leukocytes may be therapeutically useful by reducing the number of infiltrating cells in chronic inflammation without significantly depleting peripheral blood leukocytes. Eliminating CCR5-positive leukocytes from the inflammatory infiltrate should be of greater therapeutic benefit than simply blocking chemokine receptors of these cells, as they have already infiltrated the tissue.

Besides its role in inflammation, CCR5 is the primary coreceptor for M-tropic HIV-1 strains that predominate early in the course of an infection (13, 14). Transmission of HIV-1 depends on the presence of CCR5, as individuals with a homozygous Δ32 deletion of the CCR5 allele are highly resistant against infection with HIV-1 (11). Although antiretroviral therapy can efficiently suppress replication of HIV-1, complete eradication of HIV has not been achieved to date. The main obstacle appears to be the inactivity of antiretroviral therapy against latently infected cells that can survive for several years and function as an endogenous source for HIV-1 (15). Many of these cells fail to express viral proteins and can evade the immune response. However, the majority of latently infected cells may still express CCR5, as this receptor was necessary for their initial infection. We therefore propose that depletion of CCR5-positive cells should significantly reduce the number of latently infected cells in HIV-1 infection. Other strategies to eliminate HIV-1-infected cells that depend on a specific recognition of viral proteins, e.g., surface-expressed gp120 (16), would be less effective against latently infected cells.

To test these possibilities, we designed two strategies to destroy CCR5-positive cells. First, we constructed a bispecific single-chain Ab that binds with one arm to CCR5 and with the other arm to

*Medical Policlinic, University of Munich, Munich, Germany; and †Institute for Animal Physiology, University of Munich, Munich, Germany

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[1] Address correspondence and reprint requests to Dr. Matthias Mack, Medical Policlinic, University of Munich, Pettenkoferstrasse 8a, 80336 Munich, Germany. E-mail address: mack@medpoli.med.uni-muenchen.de

[2] Abbreviations used this paper: CCR5-Δ32/Δ32, homozygous 32-bp deletion in the CCR5 alleles; CHO, Chinese hamster ovary; PE38, truncated version of *Pseudomonas* exotoxin A; CXCR, CXC chemokine receptor.

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CD3 expressed on T lymphocytes. Thereby the bispecific Ab induces a close contact between CCR5-positive target cells and CD3-positive T cells. Cross-linkage of CD3 by the Ab triggers the cytotoxic activity of T lymphocytes and leads to lysis of CCR5-positive cells (Fig. 1). Bispecific Abs were originally developed for oncological therapy to redirect cytotoxic T cells against malignant cells (17). Various types of bispecific Abs have been described so far. Although the chemical cross-linkage of two mAbs and the hybrid-hybridoma technology (18) is hampered by ill-defined protein aggregates and low yields, diabodies and bispecific single-chain Abs have overcome these problems (19–23). In 1995, we described functional expression of bispecific single-chain Abs in Chinese hamster ovary (CHO) (3) cells (20, 24). These Abs are composed of two single-chain Fv fragments that are joined by a flexible linker consisting of glycine and serine molecules. We now propose a new application for bispecific Abs, namely, elimination of infiltrating leukocytes in chronic inflammation. The CCR5-CD3-bispecific Ab would specifically deplete CCR5-positive cells and reduce considerably the number of cells that infiltrate inflamed tissue. T cells and monocytes that are not involved in the inflammation would largely remain unaffected because they rarely express CCR5.

In a second approach to destroy chemokine receptor-positive cells, we designed a fusion protein of the chemokine RANTES and a truncated version of the *Pseudomonas* exotoxin A. Several fusion proteins with a truncated version of *Pseudomonas* exotoxin A have been designed so far. Most of them have been used to target and destroy malignant cells (25). A truncated version of the toxin (PE38) was used for the construct (26), as the full-length protein binds with its first domain to the ubiquitous α_2-macroglobulin receptor and is therefore toxic to most eukaryotic cells (27). To overcome this problem, the first domain of *Pseudomonas* exotoxin A can be replaced by a specific sequence to alter the binding specificity of the toxin (26). We investigated whether a chemokine can serve as ligand sequence on the toxin, direct it to specific targets by binding to the specific chemokine receptor, and induce uptake of the toxin and lysis of chemokine receptor-positive cells. We considered chemokines to efficiently mediate the uptake of the toxin as chemokine receptors are rapidly internalized upon binding of chemokines (28, 29). In this study, we show that in fact the chemokine RANTES fused to PE38 binds to CCR5 and efficiently destroys CCR5-positive target cells. Thus, both the bifunctional CD3-CCR5 Abs and the RANTES-*Pseudomonas* A toxin constructs are promising candidates as therapeutic agents.

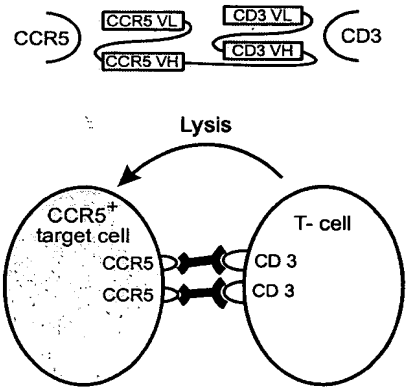

FIGURE 1. Scheme of the bispecific single-chain Ab. The anti-CCR5 single-chain fragment (CCR5 V_L/CCR5 V_H) derived from the hybridoma MC-1 is fused to the N terminus of a single-chain fragment directed against CD3 (CD3 V_H/CD3 V_L). Binding of the bispecific Ab to $CD3^+$ T cells and CCR5-positive target cells results in cross-linkage of CD3, activation of effector T cells, and lysis of CCR5-positive target cells.

Materials and Methods

PBMC, synovial fluid, and cell lines

PBMC were isolated from buffy coats or full blood of healthy donors by Ficoll density gradient centrifugation. Where indicated, PBMC were used from donors with a homozygous 32-bp deletion in the CCR5 allele (CCR5-Δ32/Δ32) preventing surface expression of CCR5. Synovial fluid of patients with arthritis was obtained from diagnostic or therapeutic arthrocentesis and used for the experiments without further preparation. Informed consent was obtained from all patients.

CHO cells were stably transfected with CCR5 or CXC chemokine receptor (CXCR) 4 as described previously (28).

Construction and expression of the bispecific single-chain Ab anti-CCR5-anti-CD3

The light (V_L) and heavy (V_H) variable domains from the anti-CCR5 hybridoma MC-1 were cloned using PCR amplification (30). Reverse transcription was conducted with random hexamer nucleotides and SuperScript reverse transcriptase (Life Technologies, Grand Island, NY). The variable domains were amplified by PCR with Pfu-polymerase, subcloned into the vector PCR-script Amp SK^+ (Stratagene, La Jolla, CA), and sequenced. As described previously, the light and heavy variable domains were joined to a single-chain fragment using a $(Gly_4Ser_1)_3$ linker and expressed in the periplasmic space of *Escherichia coli* to test binding of the recombinant protein to CCR5. Subsequently, the DNA sequence of the anti-CCR5 single-chain fragment was subcloned with BsrG1 and BspE1 into an eukaryotic expression vector (pEF-DHFR) that contained a single-chain fragment directed against CD3 with a C-terminally attached tail of six histidine residues (20). The anti-CCR5 and anti-CD3 single-chain fragments were joined by a linker coding for Gly_4Ser_1. The bispecific Ab was expressed in DHFR-deficient CHO cells and purified from the culture supernatant by affinity chromatography on immobilized Ni^{2+} ions (Ni-NTA; Qiagen, Chatsworth, CA).

Construction and expression of RANTES-PE38

A PCR fragment of RANTES, generated with the primers P1 and P2, was subcloned with *Stu*I and *Sal*I into a vector for periplasmic expression in *E. coli* (20). The restriction site *Stu*I had previously been introduced at the 3′ terminus of the OmpA signal sequence. The DNA of a truncated version of Pseudomonas exotoxin A (PE38), kindly provided by I. Pastan (26), was amplified by PCR with Pfu-polymerase using the primers P3 and P4 and subcloned with *Bsp*E1 and *Hin*dIII into the vector that already contained the cDNA of RANTES. Primer P4 also added a tail of 6 histidine residues at the 3′ terminus of PE38. During the periplasmic expression, the OmpA signal sequence is cleaved off such that the recombinant protein starts with the first amino acid of RANTES. The C-terminally attached tail of six histidine residues allowed purification by affinity chromatography on Ni-NTA (Qiagen). List of primers is as follows: P1, 5′-AAAGGCCTC CCCATATTCCTCGGA; P2, 5′-AAAGTCGACTCCGGACATCTCCAAAGA GTTGATGTAC; P3, 5′-AATCCGGAGGCGGCAGCCTGGCCGC; and P4, 5′-GGGAAGCTTAGTGATGGTGATGGTGATGCTTCAGGTCCTCGCGCGG.

FACS analysis and Western blot

Binding of the bispecific single-chain Ab to CHO cells or PBMC was determined by FACS analysis. The cells were incubated with the bispecific Ab for 60 min on ice followed by an Ab against 6xHis (Dianova, Hamburg, Germany) and a PE-conjugated polyclonal rabbit anti-mouse $F(ab')_2$ fragment (R439; Dako, Hamburg, Germany).

Western blots to detect the bispecific Ab or RANTES-PE38 were stained with the mAb against 6xHis (Dako) and a peroxidase-labeled polyclonal rabbit anti-mouse Ab (P260; Dako). Western blots to detect CCR5 in the cell-free supernatant were performed as previously described (31).

Down-modulation of chemokine receptors

PBMC were incubated for 30 min at 37°C with various concentrations of RANTES or RANTES-PE38 diluted in RPMI 1640 with 10% FCS in a volume of 100 μl. Medium alone was used as control. The cells were then stained on ice for surface CCR5 expression using the mAb MC-1 or medium as negative control followed by the PE-conjugated anti-mouse Ab R439. As shown previously, the presence of RANTES bound to CCR5 does not block the binding of MC-1 to CCR5 (28). The same could be

demonstrated for RANTES-PE38. The FACS analysis was performed on a FACSCalibur (Becton Dickinson, Mountain View, CA) and CellQuest software. Lymphocytes and monocytes were distinguished by their forward and sideward light scatter properties and expression of CD14, CD4, and CD8. Relative surface CCR5 expression was calculated as [mean channel fluorescence (experimental) − mean channel fluorescence (negative control)]/[mean channel fluorescence (medium) − mean channel fluorescence (negative control)].

Depletion of cells with the bispecific anti-CCR5-anti-CD3 Ab and RANTES-PE38

PBMC from CCR5 wild-type or CCR5-deficient (Δ32/Δ32) donors were incubated overnight to induce expression of CCR5 on monocytes. Cultured PBMC or freshly drawn synovial fluid of patients with arthritis were incubated with different concentrations of purified anti-CCR5-anti-CD3-bispecific Abs or medium as control for 20 h. No preactivation of effector T cells was performed. Surviving cells were analyzed on a FACSCalibur and counted.

CHO cells expressing CCR5 or CXCR4 were grown to subconfluence on 24-well culture plates and incubated with different concentrations of purified RANTES-PE38 or medium as control. After 40 h, the adherent and nonadherent cells were recovered and analyzed by FACS to measure the percentage of dead cells. We have previously established that dead (propidium iodide-positive) CHO cells can be identified by their light scatter properties.

Results

Production of a bispecific single-chain Ab directed against CCR5 and CD3

As described in *Materials and Methods*, we amplified the DNA sequences coding for the variable domains of the light (V_L) and heavy chain (V_H) of the CCR5-specific hybridoma MC-1 by RT-PCR. Subsequently, we constructed a single-chain fragment by joining the V_L and V_H sequences with a linker coding for $(Gly_4Ser_1)_3$ and expressed it in the periplasmic space of *E. coli* to test the binding activity of the construct. To obtain the bispecific single-chain Ab, we joined the DNA sequence of the CCR5 Ab fragment to an Ab fragment directed against CD3 using a linker coding for Gly_4Ser_1. The bispecific single-chain Ab was expressed in CHO cells and purified via a C-terminally attached histidine tail with an overall purification yield of ~900 μg/L culture supernatant. SDS-PAGE showed a single band of ~60 kDa under reducing and nonreducing conditions without any detectable proteolysis or degradation of the protein (Fig. 2).

Binding of the bispecific Ab to CCR5 and CD3

Binding of the bispecific Ab to CD3-positive T cells was demonstrated by FACS analysis (Fig. 3). As the bispecific Ab would also bind to CCR5, we performed the analysis with PBMC that lack expression of CCR5 due to a homozygous 32-bp deletion in the CCR5 alleles. The Ab showed good binding to T cells as identified by costaining with Abs against CD4 and CD8 (Fig. 3). In addition,

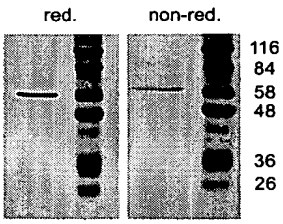

FIGURE 2. SDS-PAGE of the purified bispecific single-chain Ab anti-CCR5-anti-CD3. A single band of ~60 kDa is visible under reducing (*left*) and nonreducing (*right*) conditions. No degradation or proteolysis of the bispecific Ab is detectable.

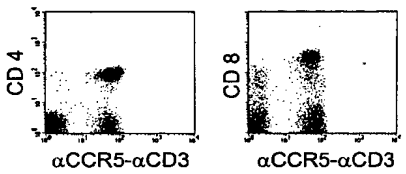

FIGURE 3. Binding of the anti-CCR5-anti-CD3-bispecific Ab to CD3 on CCR5-Δ32/Δ32 lymphocytes. Costaining with CD4 and CD8 demonstrated that the bispecific Ab binds to the subpopulation of $CD4^+/CD8^+$ T cells.

the bispecific Ab competed with the monoclonal CD3 Ab OKT-3 for binding to T cells (data not shown).

Binding of the bispecific Ab to CCR5 was demonstrated on CCR5-overexpressing CHO cells and human monocytes (Fig. 4). The Ab showed excellent binding to CCR5-transfected CHO cells (Fig. 4*A*) and cultured monocytes (Fig. 4*B*), while no binding was detectable on CHO cells transfected with CXCR4 or on cultured monocytes from a donor with a homozygous CCR5-Δ32/Δ32 deletion. Overnight cultivation of monocytes induces expression of CCR5 on wild-type monocytes, while monocytes from donors with a homozygous CCR5-Δ32/Δ32 deletion fail to express CCR5. Moreover, the CCR5 signal detectable with the bispecific Ab on cultured monocytes could be reduced to values below 15% by preincubation of monocytes for 30 min at 37°C with AOP-RANTES, a CCR5 ligand that is known to efficiently induce internalization of CCR5 (data not shown) (28). Preabsorption of the bispecific Ab on $CCR5^+$ CHO cells prevented subsequent binding to CD3 and preabsorption on CCR5-deficient T cells almost completely abolished subsequent binding to CCR5 (data not shown). This further indicates that both specificities of the bispecific Ab are contained in the same molecule.

CCR5-specific depletion of monocytes from cultured PBMC

To test the ability of the anti-CCR5-anti-CD3-bispecific single-chain Ab to deplete CCR5-positive primary cells, we incubated human PBMC with the bispecific Ab (Fig. 5). Before incubation the PBMC were cultured overnight to up-regulate CCR5 expression on monocytes (28, 32). By retargeting cytotoxic T cells, the bispecific Ab depleted the majority of monocytes within 20 h in a concentration-dependent manner (Fig. 5). In contrast to the bispecific Ab, the monovalent single-chain Fv fragments directed

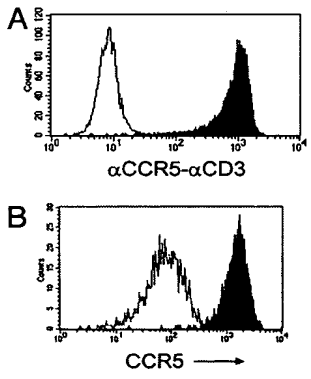

FIGURE 4. Binding of the anti-CCR5-anti-CD3-bispecific Ab to CCR5 on transfected CHO cells (*A*) and cultured monocytes (*B*). CHO cells transfected with CCR5 and monocytes from a CCR5-positive donor are shown in black, while CXCR4-positive CHO cells and monocytes from a CCR5-deficient donor (Δ32/Δ32) served as negative control and are shown in white.

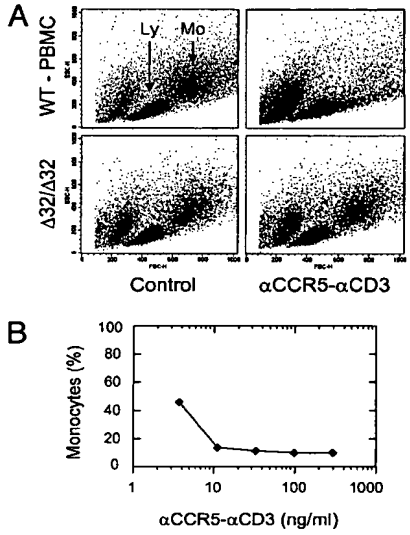

FIGURE 5. Depletion of CCR5-positive monocytes by the bispecific Ab. *A*, CCR5-deficient PBMC (Δ32/Δ32) or wild-type PBMC (WT-PBMC) were cultured overnight and incubated with the bispecific Ab (100 ng/ml) or medium as control for 20 h. Remaining monocytes (Mo) and lymphocytes (Ly) were identified by their light scatter properties in FACS. The CCR5-positive wild-type monocytes were completely depleted by the bispecific Ab, whereas the CCR5-deficient monocytes survived. *B*, Dose response showing depletion of cultured monocytes with various concentrations of the anti-CCR5-anti-CD3-bispecific Ab. More than 90% of the monocytes were depleted at a concentration of 33 ng/ml.

against CCR5 and CD3 used either alone or in combination were not able to deplete CCR5-positive monocytes (data not shown). To verify that the depletion of monocytes with the bispecific Ab was due to their expression of CCR5, we performed the same experiment with PBMC from a donor with a homozygous 32-bp deletion in the CCR5 allele that prevents surface expression of CCR5. No depletion of CCR5-deficient monocytes occurred after 20 h, indicating that the depletion of cells with the bispecific Ab is restricted to monocytes that express CCR5 (Fig. 5*A*). As further control, we preincubated PBMC with an excess of the parental mAb MC-1 or a control Ab and then added the bispecific Ab. Preincubation with MC-1 considerably reduced the depletion of monocytes while the control Ab had no effect (data not shown).

Depletion of monocytes and T lymphocytes from the synovial fluid of patients with arthritis

The bispecific single-chain Ab could potentially be applied to deplete CCR5-positive T cells and monocytes from the inflamed joints of patients with arthritis. We therefore determined the depletion of CCR5-positive cells from the synovial fluid of patients with various types of arthritis. Before each depletion experiment, we confirmed by FACS analysis that the majority of lymphocytes and monocytes in the synovial samples express CCR5, whereas no expression of CCR5 was detectable on granulocytes (data not shown). The synovial fluid was incubated ex vivo with different concentrations of the bispecific Ab immediately after arthrocentesis without prior preparation or washing steps. Using freshly drawn synovial fluid, we aimed at testing the efficacy and stability of the bispecific Ab under conditions resembling most closely the situation in vivo. Within 20 h, the bispecific Ab induced depletion of the majority of lymphocytes and monocytes from the synovial fluid, while granulocytes that do not express CCR5 remained unaffected (Fig. 6).

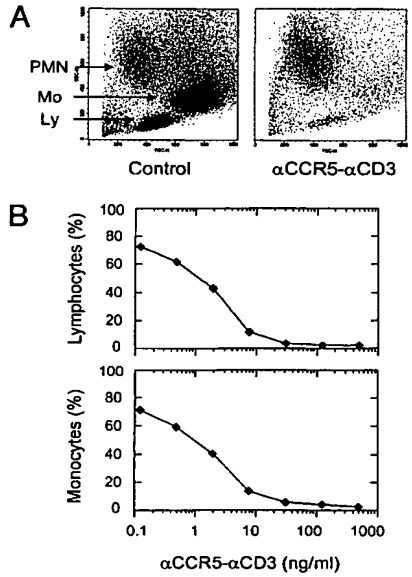

FIGURE 6. The bispecific anti-CCR5-anti-CD3 Ab depletes lymphocytes and monocytes from the synovial fluid of a patient with chronic arthritis. *A*, Freshly draw synovial fluid was incubated with the bispecific Ab (500 ng/ml) or medium as control for 20 h and analyzed by FACS. The bispecific Ab completely depleted the CCR5-positive monocytes and lymphocytes, whereas the CCR5-negative granulocytes (PMN) survived. Consistent with our previous data, all monocytes and lymphocytes in this synovial fluid expressed CCR5, whereas no expression of CCR5 was found on granulocytes (data not shown). *B*, Dose response for the depletion of monocytes and lymphocytes from the synovial fluid with the anti-CCR5-anti-CD3-bispecific Ab. More than 95% of both cell types were depleted at a concentration of 31 ng/ml.

Construction of the chemokine toxin RANTES-PE38

As described in *Materials and Methods*, the DNA sequence of RANTES was fused with the sequence of a truncated version of the *Pseudomonas* exotoxin A (PE38) kindly provided by I. Pastan (26). In a first version of the construct, a Gly-Ser linker was spaced between RANTES and PE38. However, this resulted in a considerable proteolytic degradation of the fusion protein during expression in *E. coli* (data not shown). To stabilize the construct, we removed the linker and the first three amino acids of PE38. The new fusion protein showed no proteolysis during expression in the periplasmic space of *E. coli* as demonstrated by SDS-PAGE and Western blot (Fig. 7).

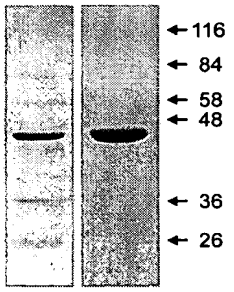

FIGURE 7. SDS-PAGE (*left*) and Western blot (*right*) of the purified protein RANTES-PE38. A distinct band with the expected size of ~46 kDa is visible in the Coomassie-stained SDS-PAGE and Western blot. Most likely our affinity purification is not 100%, which explains the faint bands in the Coomassie stain.

Biologic activity of the RANTES-PE38 construct

The fusion of RANTES to the N terminus of a truncated version of the *Pseudomonas* exotoxin A is supposed to result in specific binding of the construct to cells expressing RANTES receptors such as CCR5, CCR1, and CCR3. Internalization of the chemokine receptors upon binding of the modified toxin would enhance the cellular uptake and cytotoxic activity of the construct. We therefore analyzed whether RANTES-PE38 is able to internalize CCR5 from the surface of primary monocytes and T cells (Fig. 8). Internalization of CCR5 would indicate that the construct is able to bind to CCR5 and that RANTES remains functionally active after fusion to PE38. As shown in Fig. 8, the construct is able to internalize CCR5 from the surface of monocytes and lymphocytes. Compared with unmodified RANTES, the chemokine toxin was somewhat less effective in down-modulating CCR5. To exclude the possibility that RANTES-PE38 increases the shedding of CCR5 from the cell surface and thereby reduces surface expression of CCR5, we quantified by Western blot the amount of CCR5 in the cell-free supernatant after incubation of CCR5-positive CHO cells for 30 min at 37°C with unmodified RANTES, RANTES-PE38, or medium alone. Compared with the medium control, no increased shedding of CCR5 was detectable with either RANTES or RANTES-PE38, indicating that the reduced surface expression is due to internalization of CCR5.

We then analyzed the cytotoxic activity of RANTES-PE38. For that purpose, we incubated CHO cells expressing human CCR5, murine CCR5, and human CXCR4 with various concentrations of the chemokine toxin or medium. No surviving (i.e., adherent) human or murine CCR5-positive CHO cells were detectable by light microscopy after a 40-h incubation with as little as 10 nM RANTES-PE38. In contrast, regular growth and survival was observed when the CCR5-positive cells were incubated with medium or when CXCR4-positive CHO cells were incubated with equal concentrations of the chemokine toxin (data not shown). To quantify the percentage of dead cells, we analyzed the adherent and nonadherent cells by FACS. We have previously established that living and dead CHO cells can be identified by their light scatter properties. As shown in Fig. 9*A*, no cytotoxic effect of RANTES-PE38 was seen on CHO cells expressing CXCR4, whereas CHO cells expressing human CCR5 were completely killed by 10 nM RANTES-PE38. As further control, we preincubated $CCR5^+$ CHO cells with 10 μg/ml unconjugated RANTES and then added the chemokine toxin (Fig. 9*B*). Preincubation of the $CCR5^+$ cells with unconjugated RANTES completely prevented their destruction by RANTES-PE38. RANTES alone did not influence the viability of the cells.

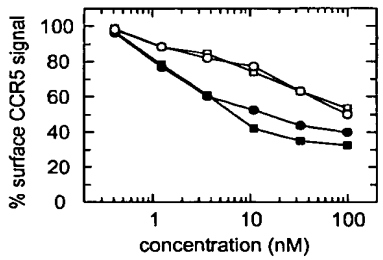

FIGURE 8. Down-modulation of CCR5 from the surface of PBMC by incubation with RANTES-PE38 (○, □) and RANTES (●, ■) for 30 min at 37°C. Surface expression of CCR5 was determined on lymphocytes (□, ■) and monocytes (○,●) and is given as percentage of the medium control. The fusion protein RANTES-PE38 is able to down-modulate CCR5 from the cell surface with a somewhat lower efficiency than unmodified RANTES.

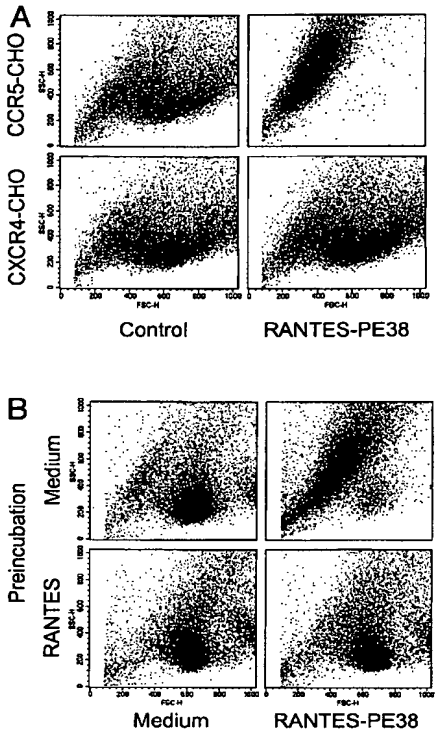

FIGURE 9. Destruction of CCR5-positive CHO cells with the chemokine toxin RANTES-PE38. *A*, CCR5-positive CHO cells and CXCR4-positive CHO cells were incubated for 40 h with the chemokine toxin (10 nM) and analyzed by FACS. Dead cells appear in the *left upper region* of the forward and sideward light scatter plot. RANTES-PE38 completely destroyed the CCR5-positive CHO cells, whereas it had no effect on the CXCR4-positive CHO cells. *B*, The cytotoxic activity of RANTES-PE38 could be completely blocked by addition of unmodified RANTES (10 μg/ml) 60 min before incubation with RANTES-PE38. Preincubation with medium served as control and did not prevent the killing of cells.

These experiments indicate that RANTES-PE 38 is able to internalize CCR5 from the surface of cells and induces depletion of cells expressing the RANTES receptors human CCR5 or murine CCR5. The inactivity of the construct against CXCR4-positive CHO cells demonstrates that the cytotoxic activity of the construct is restricted to cells that express specific chemokine receptors.

Discussion

We propose that a specific depletion of CCR5-positive cells may be therapeutically useful in chronic inflammatory diseases and HIV-1 infection. In chronic inflammation such as rheumatoid arthritis (4, 5), inflammatory renal diseases including transplant rejection (6), multiple sclerosis (8, 9), and inflammatory bowel disease (10), a clear predominance of T cells and monocytes expressing the chemokine receptor CCR5 is found within the affected tissues. In contrast, in the peripheral blood only a minority of T cells and monocytes express CCR5 (4). A depletion of CCR5-positive cells may therefore considerably reduce the infiltrate in the inflamed tissue while other leukocytes would remain largely unaffected. Also, identification of latently HIV-infected cells by CCR5 and their specific depletion could be a clinical application of the bispecific Abs and chemokine toxins.

We present two possibilities to deplete CCR5-positive cells. First, we describe a bispecific single-chain Ab that simultaneously binds to CCR5 and CD3 and thereby redirects T effector cells

against CCR5-positive target cells (Fig. 1). A precondition for the efficacy of this bispecific Ab is the presence of T effector cells. In rheumatoid arthritis, T cells are enriched in the synovial fluid and tissue and their percentage further increases with disease duration. Since most of these T cells are CCR5 positive, they would deplete each other as shown for the synovial fluids of patients with arthritis (Fig. 6). Many of the infiltrating T cells potentially recognize autoantigens and therefore perpetuate the disease process. In chronic glomerulonephritis, interstitial nephritis, and transplant rejection, CCR5-positive T cells are abundantly present in areas of interstitial infiltration and their number correlates with the degree of renal insufficiency (6). Apart from T cells, CCR5 is expressed by the majority of monocytes/macrophages within the inflamed tissue. Monocytes/macrophages are responsible for destruction of inflamed tissue. The depletion of infiltrating monocytes/macrophages may therefore considerably reduce the tissue damage, e.g., joint destruction in rheumatoid arthritis. Moreover, CCR5 is highly expressed on dendritic cells (33) that are found in the synovial fluid and synovial tissue in rheumatoid arthritis and are thought to play a major role for initiation and perpetuation of the inflammatory process (34).

A specific depletion of CCR5-positive cells appears to have several advantages over conventional immunosuppressive or immunomodulatory therapy: 1) Depletion of infiltrating cells would only take a couple of hours, while immunomodulatory agents usually need several weeks for their onset of action. 2) Conventional treatments only suppress the activity of infiltrating cells which leads to a rapid relapse after termination of the therapy. In contrast the bispecific Ab would eliminate the infiltrating cells and may therefore exert a prolonged benefit. 3) Elimination of CCR5-positive leukocytes is supposed to be well tolerated as CCR5-positive cells are enriched in the inflamed tissue and only rarely encountered in the peripheral blood. A local application of bispecific Abs, e.g., intra-articular injection in arthritis, would further reduce potential side effects.

Chemokine toxins significantly differ from bispecific Abs in their mechanism of depletion. Although bispecific Abs depend on T effector cells to deplete other cells, the mere binding and internalization of chemokine toxins is sufficient to induce cell death. Binding and internalization of chemokine toxins depends on the expression of appropriate chemokine receptors. In many cases, chemokines bind to more than one receptor, which would result in a more extensive destruction of cells. The chemokine toxin RANTES-PE38 binds to CCR5 and eliminates CCR5-positive CHO cells. However, we assume that the construct would also recognize and destroy CCR1- or CCR3-positive cells since RANTES also binds to these receptors (35). The main advantage of chemokine toxins is their ease of production, as the chemokine moiety can be rapidly exchanged by one cloning step. For most receptors, one would also be able to select a chemokine that only binds to one receptor. Chemokine toxins would therefore be an ideal tool to study depletion of selected subtypes of cells in various animal models of inflammation.

The expression of chemokine receptors on leukocytes is associated with certain types of an immune response. Recently, it was described that the receptors CCR5 and CXCR3 are primarily expressed on TH-1 cells, while CCR3, CCR4, and CCR8 are mainly found on TH-2 cells (36–39). Depletion of chemokine receptor-positive cells may therefore induce an immune deviation from TH-2 to TH-1 or vice versa. Depletion of CCR3-positive cells may be of benefit in allergic diseases, as CCR3 is not only expressed on TH-2 cells but also on eosinophils and basophils (40, 41).

Apart from chronic inflammatory diseases, the depletion of CCR5-positive cells could also be useful for the treatment of HIV-1 infection to reduce the number of latently infected cells. Early in the course of an HIV infection, preferentially CCR5-positive cells are infected with HIV-1 (14). A small fraction of these cells, mainly $CD4^+$ T cells, macrophages, and potentially dendritic cells, become latently infected and function as an endogenous source for HIV-1 (15). Latently infected cells can survive for several years and are thought to be responsible for the failure to completely eradicate HIV-1 despite prolonged highly active antiretroviral therapy. We therefore propose that depletion of $CCR5^+$ cells would shorten the half-life of latently infected cells and could help to eradicate HIV-1 in combination with highly active antiretroviral therapy. Latently infected cells cannot be identified by surface expression of viral proteins, as little viral genes are expressed when the virus is dormant. CCR5 however would still be expressed on latently infected cells as it was necessary for initial infection.

Specific depletion of chemokine receptor-positive cells can be achieved with bispecific Abs and chemokine toxins and may represent a new strategy in the treatment of chronic inflammatory diseases and HIV-1.

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Intrapulmonary targeting of RANTES/CCL5-responsive cells prevents chronic fungal asthma

Jane M. Schuh[1], Kate Blease[1], Hilke Brühl[2], Matthias Mack[2] and Cory M. Hogaboam[1]

[1] Department of Pathology, University of Michigan Medical School, Ann Arbor, USA
[2] Medical Policlinic, Ludwig-Maximilians-University Munich, Munich, Germany

Regulated upon activation in normal T cells, expressed, and secreted (RANTES)/CCL5 is abundantly expressed during atopic asthma, suggesting that it is an important mediator of this disease. The contribution of intrapulmonary RANTES/CCL5-sensitive cells during *Aspergillus fumigatus*-induced airway disease in mice was assessed in this study. The intranasal delivery of a chimeric protein comprised of RANTES/CCL5 and a truncated version of *Pseudomonas* exotoxin A (RANTES-PE38) significantly attenuated serum IgE, peribronchial eosinophilia, and airway hyperreactivity when it was administered from day 0 to 15 after intratracheal conidia challenge in *A. fumigatus*-sensitized mice but had little effect when delivered from day 15 to 30 after conidia challenge. Intranasal RANTES-PE38 treatment enhanced macrophage recruitment and accelerated fungal clearance in the lungs of RANTES-PE38-treated mice. These data reveal a major role for RANTES/CCL5 and its receptors in the development of fungal asthma yet reveal only a modest role in the chronic remodeling of the allergic airway in this disease.

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1 Introduction

Chemokines are low-molecular-weight molecules that recruit leukocytes to sites of inflammation and infection via G-protein-linked receptors. Although originally described as a T cell-specific CC chemokine [1], RANTES/CCL5 also affects the directed migration and/or activation of monocytes [2] and eosinophils [3]. RANTES/CCL5 is produced by a number of resident and circulating cells [4–10]. RANTES/CCL5 may activate cells by binding to CC chemokine receptor-1 (CCR1), CCR3, or CCR5 [11], which it shares with other chemokines.

RANTES/CCL5 is among the major CC chemokines induced during atopic [12] and nonatopic [13] asthmatic responses in clinical asthma [14]. The severity of asthma and atopy has been associated with a polymorphism in the RANTES/CCL5 promoter (–403 G→A) [15]. RANTES/CCL5 has also been shown to enhance the production of IgE and IgG4 by activated B cells [16]. In addition, RANTES/CCL5 recruits eosinophils and T cells into the airways during asthma [17] and allergic airway disease [18] and may contribute to their immunopathology [19, 20]. Eosinophils cause tissue damage and promote allergic disease in the lung through the release of toxic proteases, lipid mediators, cytokines, and oxygen free radicals [21], whereas T cells are a source of pro-allergic cytokines including IL-4 and IL-13 [22]. In experimental studies, RANTES/CCL5 recruits T cells and eosinophils during *Schistosoma* egg antigen- [23] and ovalbumin (OVA)- [24] induced allergic airway disease.

The present study was designed to define the role of RANTES/CCL5-sensitive lung cells during the development and maintenance of allergic airway disease induced by the introduction of *Aspergillus fumigatus* conidia (or spores) into mice sensitized to this fungus. An immunotoxin, RANTES-PE38, was used to target RANTES/CCL5-responsive cells. RANTES-PE38 is a chimeric protein composed of intact RANTES/CCL5 DNA fused to the N terminus of a truncated form of *Pseudomonas* exotoxin A (PE38). RANTES/CCL5 retains its functional ability to bind to its receptors – CCR5, CCR3, and CCR1 – and the toxin is specifically internalized through these receptors. The toxin cannot bind independently of the chemokine. This specificity has been demonstrated in Chinese hamster ovary (CHO) cells transfected with

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Abbreviations: RANTES: Regulated upon activation in normal T cells, expressed, and secreted **RANTES-PE38:** RANTES/CCL5-*Pseudomonas* exotoxin A chimeric protein **BAL:** Bronchoalveolar lavage **GMS:** Gomori methenamine silver **MDC:** Macrophage-derived chemokine **TARC:** Thymus and activation-related chemokine

CCR5 [25]. Although chemotaxis cannot be formally tested *in vitro* while the chemokine is attached to the toxin moiety, the RANTES/CCL5 portion of the immunotoxin presumably retains its chemotactic function. Experimental data presented in this manuscript suggest that RANTES-PE38 can both enlist and kill recruited cells.

Here, *A. fumigatus* was used to sensitize mice in a model of fungal allergic airway disease. Immune responses to *A. fumigatus* can exacerbate atopy and asthma [26]. Chronic airway remodeling in these individuals often leads to a poor clinical outcome. The allergic response to *Aspergillus* involves a number of immunological abnormalities including elevated IgE, enhanced Th2 cytokines [27], eosinophilic and T cell inflammation, and profound airway remodeling [28]. Early in the *A. fumigatus*-induced fungal asthma model, CCR5 is the major receptor for RANTES/CCL5. Previous research has shown that while RANTES/CCL5 can bind CCR1 and/or CCR3 in the absence of CCR5 [29], CCR5 ablation alters the dynamics of receptor utilization drastically. CCR1 is expressed much earlier in the $CCR5^{-/-}$ mouse (unpublished observation). Ostensibly, this up-regulation is triggered by the abnormal kinetics created by the absence of CCR5. The reduction in $CCR5^{+}$ cells was used to assess the efficiency of the immunotoxin treatment (appropriate reagents were unavailable for CCR1 and CCR3). This research demonstrates that the selective targeting of RANTES/CCL5-responsive cells with a chimeric immunotoxin may offer a promising therapeutic approach in allergic and asthmatic diseases.

2 Results

2.1 Whole-lung levels of RANTES/CCL5 protein are increased following conidia challenge in *A. fumigatus*-sensitized mice

We examined RANTES/CCL5 levels in whole-lung samples during the course of chronic allergic airway disease due to *Aspergillus*. Several time points were examined to track temporal changes in RANTES/CCL5 production over the 30 days following intratracheal introduction of 5×10^6 *A. fumigatus* conidia into *A. fumigatus*-sensitized mice. ELISA analysis is shown in Fig. 1. At all points examined, whole-lung levels of RANTES/CCL5 were significantly ($p\leq0.05$) increased above baseline levels (Fig. 1). The highest levels of RANTES/CCL5 were detected at day 8 after conidia challenge, but elevated levels could be observed more than 30 days post-conidia challenge. Thus, these data show that whole-lung levels of RANTES/CCL5 were increased and remained increased in *A. fumigatus*-sensitized mice after an intratracheal challenge with *A. fumigatus* conidia.

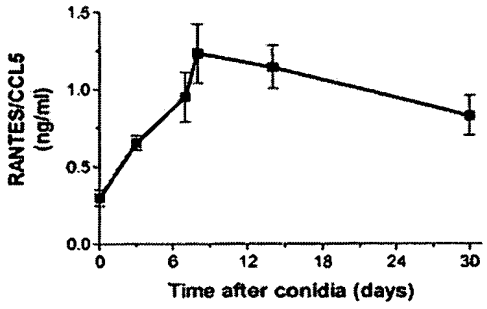

Fig. 1. Whole-lung levels of RANTES/CCL5 in *A. fumigatus*-sensitized CBA/J mice prior to and at days 3, 7, 8, 14, and 30 after *A. fumigatus* conidia challenge, as measured by ELISA. Data are expressed as mean ± SEM; n=5/group/time point.

2.2 Early RANTES-PE38 treatment significantly attenuated airway hyperresponsiveness in mice with *Aspergillus*-induced allergic airway disease

With both clinical and experimental data verifying that RANTES/CCL5 is increased significantly in instances of atopy, we addressed whether targeting RANTES/CCL5-responsive cells in the lung could prevent the appearance of or reverse the features of this disease. RANTES/CCL5-sensitive cells were targeted by the intranasal instillation of RANTES-PE38, a novel chemokine toxin that had previously been shown to kill CCR5-positive cells *in vitro* [25]. Airway hyperreactivity in RANTES-PE38- and control-treated mice at days 15 and 30 after conidia challenge is shown in Fig. 2. When *A. fumigatus*-sensitized CBA/J mice challenged with conidia received RANTES-PE38 from day 0 to 15 after conidia, airway hyperresponsiveness to a systemic methacholine challenge was significantly lower than that measured in similarly sensitized and challenged control mice that received the same dose of methacholine (Fig. 2A). However, when mice were treated from day 15 to 30 after conidia challenge, airway hyperresponsiveness was not significantly attenuated (Fig. 2B). These data suggested that the early targeting of RANTES/CCL5-responsive cells in the lungs prevents the development of airway hyperreactivity during chronic allergic airway disease.

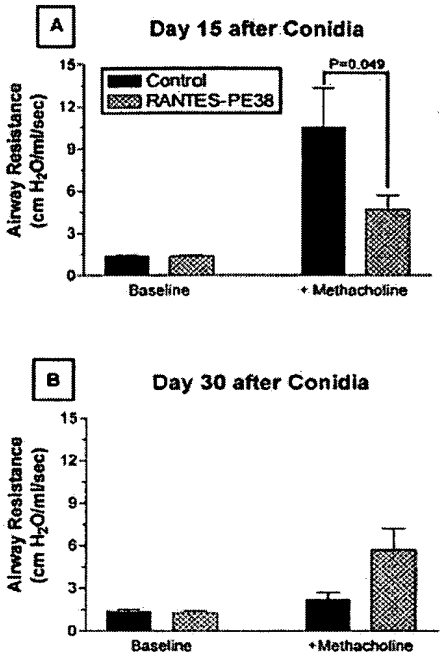

Fig. 2. Airway hyperresponsiveness in *A. fumigatus*-sensitized CBA/J mice at day 15 (A) or 30 (B) after an intrapulmonary challenge with live *A. fumigatus* conidia. In the day-15 study (A), each mouse received 1 μg of RANTES-PE38 in saline or saline alone intranasally 2 h before conidia challenge and every 48 h after conidia challenge for 15 days. In the day-30 study (B), each mouse received 1 μg of RANTES-PE38 in saline or saline alone intranasally at day 15 after the conidia challenge and every 48 h thereafter until day 30 after conidia challenge. Peak increases in airway hyperresponsiveness (cm H_2O/ml/s) were determined at each time point with intravenous injection of methacholine. Values are expressed as mean ± SEM; n=5–10/group/time point.

2.3 RANTES-PE38 treatment significantly inhibited peribronchial inflammation

Representative histological lung sections from control and RANTES-PE38-treated mice at day 15 after conidia challenge are shown in Fig. 3. Mice that received the RANTES/CCL5 chimeric protein (Fig. 3B) over the first 15 days of this model had less peribronchial inflammation than control mice (Fig. 3A, arrow). Histological analysis of whole-lung sections from mice treated with saline (Fig. 3C) or RANTES-PE38 (Fig. 3D) from days 15 to 30 after conidia challenge revealed a similar reduction in peribronchial inflammation at day 30; however, the RANTES-PE38-treated lungs exhibited interstitial inflammation to a greater degree than control lungs (Fig. 3D, arrow).

Interestingly, the diminished numbers of inflammatory leukocytes around the airways at day 15 did not translate to decreased numbers of leukocytes in the bronchoalveolar lavage (BAL) fluid at the same time point (Fig. 4). Morphometric analysis revealed that significantly more eosinophils and macrophages were present in the BAL fluid of the RANTES-PE38 group treated from day 0 to 15 (Fig. 4A) as compared with controls. In contrast, mice treated from day 15 to 30 after conidia challenge (Fig. 4B) had numbers nearly equivalent to controls in all populations examined. These data suggested that RANTES/CCL5 recruits cells even when bound to the toxin. It also appeared that RANTES/CCL5-sensitive cells dictate the regulation of early disease features, and the removal of this regulation had profound effects on the disease outcome.

2.4 Fungal elements are cleared more rapidly in RANTES-PE38-treated lungs

Gomori methenamine silver (GMS)-stained whole-lung sections from mice treated with normal saline or RANTES-PE38 were examined at day 15 or 30 after the conidia challenge to determine differences in clearance of the fungus (Fig. 5). The GMS stain appears black in the presence of polysaccharide components from the fungus cell wall (arrow, black dots). Staining shows a marked reduction of fungal elements in mice that were treated with RANTES-PE38 from days 0 to 15 after conidia challenge (Fig. 5B) when compared with control sections (Fig. 5A). A reduction in fungal elements is also seen in the animals that were treated from days 15 to 30 after conidia challenge (Fig. 5D) when staining was compared with controls (Fig. 5C), although this reduction is less dramatic. Interestingly, there seems to be increased matrix staining in the RANTES-PE38-treated group (black alveolar wall, Fig. 5D). Since conidia clearance is dependent primarily upon early innate immune events, it was expected that the early treatment course would have a more marked effect on conidia removal. These observations suggested that treatment with RANTES-PE38 at the initiation of fungal asthma enhanced the clearance of *A. fumigatus* conidia from the airways of challenged mice.

2.5 RANTES-PE38 treatment inhibited serum levels of total IgE

RANTES/CCL5 has been shown to enhance IgE production by B cells [16]. Correspondingly, the levels of total IgE in the serum of RANTES-PE38-treated mice at days 15 and 30 after conidia challenge were significantly lower than levels measured in the saline-treated groups

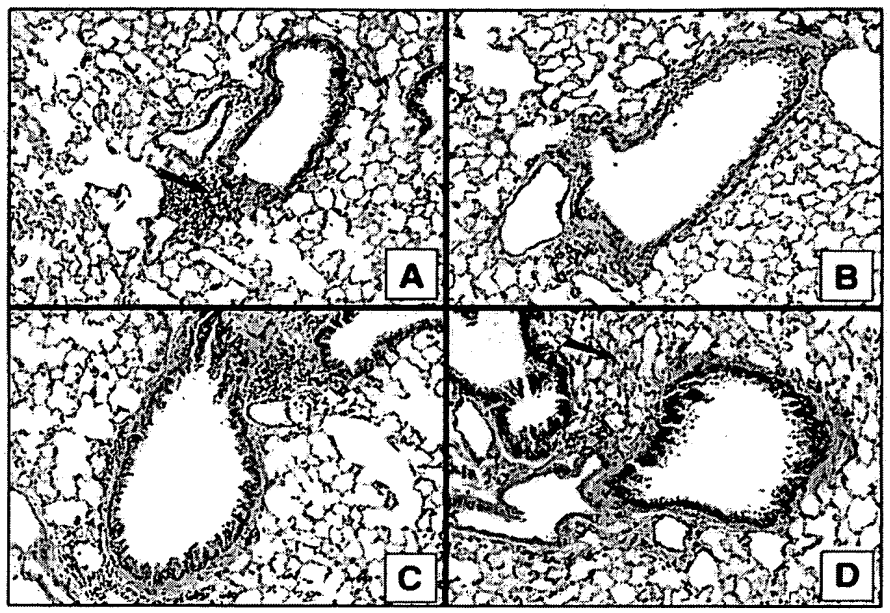

Fig. 3. Representative photomicrographs of H/E-stained whole-lung sections at days 15 (A, B) and 30 (C, D) after *A. fumigatus* conidia challenge. (A, B) Mice received saline (A) or 1 μg of RANTES-PE38 in saline (B) intranasally 2 h prior to the conidia challenge and every 48 h until day 15 after conidia challenge. (C, D) Mice received saline (C) or 1 μg of RANTES-PE38 in saline (D) intranasally at day 15 and every 48 h until day 30 after conidia challenge. Peribronchial inflammation was increased in lung sections from saline-treated mice at day 15 (A, arrow) compared with RANTES-PE38-treated mice (B). Peribronchial inflammation was reduced in the RANTES-PE38-treated mice at day 30 after conidia challenge (D) when compared to control mice (C), while interstitial inflammation was still evident in these sections (D, arrow). Original magnification was 200×.

at the same times after the conidia challenge (Fig. 6A). The RANTES-PE38 treatments did not affect the circulating levels of IgG1 (Fig. 6B). While it appeared that the RANTES-PE38 treatment increased IgG2A (Fig. 6C) at both times examined after the conidia challenge, these differences did not reach statistical significance.

2.6 RANTES-PE38 treatment significantly inhibited whole-lung levels of macrophage-derived chemokine/CCL22 and thymus and activation-related chemokine/CCL17

Whole-lung chemokine levels were examined by ELISA at days 15 and 30 after conidia challenge (Fig. 7). Unexpectedly, RANTES-PE38 treatment did neither modulate the major ligands of CCR1, CCR3, or CCR5 [RANTES/CCL5, eotaxin/CCL11, and macrophage inflammatory protein-1α (MIP-1α)/CCL3] nor alter levels of monocyte chemoattractant protein-1 (MCP-1)/CCL2 compared with those observed in control animals. However, immunotoxin treatment significantly reduced whole-lung levels of macrophage-derived chemokine (MDC)/CCL22 (days 15 and 30) and thymus and activation-related chemokine (TARC)/CCL17 (day 15) compared with controls. These data suggested that RANTES-PE38 modulated the activation of cells that generated these CCR4 (MDC/CCL22 and TARC/CCL17) and CCR8 (TARC/CCL17) ligands.

2.7 RANTES-PE38 treatment failed to attenuate goblet cell metaplasia

Goblet cell metaplasia is a prominent feature of chronic allergic airway disease [30]. Periodic acid-Schiff (PAS) staining of whole-lung sections revealed that the RANTES-PE38 treatment had no appreciable effect on the goblet cell metaplasia at either time point after conidia challenge (data not shown). This interesting observation reiterates earlier findings in $CCR4^{-/-}$ mice, in which all of the early indexes of disease were abolished but goblet cell metaplasia persisted in *Aspergillus*-sensitized mice examined 30 days after conidia challenge [31].

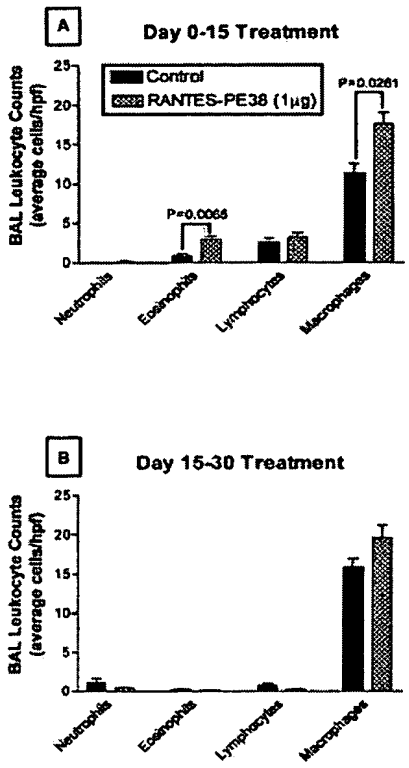

Fig. 4. BAL leukocyte counts in control and RANTES-PE38-treated mice at days 15 (A) and 30 (B) after conidia challenge in *A. fumigatus*-sensitized mice. In the day-0–15 group (A), mice received saline or 1 μg of RANTES-PE38 in saline intranasally 2 h before conidia challenge and every 48 h for 15 days. In the day-15–30 group (B), each mouse received saline or 1 μg of RANTES-PE38 in saline intranasally every 48 h from day 15 until day 30 after conidia challenge.

2.8 Flow cytometric analysis of immune cells from lung cell preparations revealed a marked reduction in F4/80$^+$ macrophages at day 15 after conidia challenge

CCR5 is expressed on a number of cell types in the lung, on both recruited and resident cells. To determine whether one particular cell type was more sensitive to the immunotoxin treatment and by association more important in this model, cell suspensions pooled from digests of control or RANTES-PE38-treated lungs were used for flow cytometric staining. There was a general reduction in the total number of CCR5$^+$ T cells (as represented by CD4$^+$ and CD8$^+$ cells), B cells (CD19$^+$), and NK cells (DX5$^+$) at both days 15 and 30 after conidia challenge when compared to control animals (Fig. 8). Intriguingly, the CCR5$^+$, F4/80$^+$ macrophage population was totally obliterated by the RANTES-PE38 treatment at day 15, yet only moderately reduced at day 30 (Fig. 8). This result was surprising given that conidia clearance was accelerated in the treated mice and more macrophages were observed in the BAL fluid at day 15.

2.9 Immunohistochemistry illustrated the marked decrease of CCR5$^+$ interstitial cells in RANTES-PE38-treated lungs

Immunohistochemical analysis was used to identify CCR5$^+$ cells *in situ* in lung sections from animals treated with normal saline or with 1 μg of RANTES-PE38 every 48 h in both the early (days 0–15) and late (days 15–30) treatment groups. Fig. 9 shows the day-15 time point: very little CCR5 was detected in the control or treated day-30 sections. Individual cells in the interstitium of control animals, probably resident macrophages, were positive for CCR5 (Fig. 9A) while treated animals had almost no immunoreactive CCR5 in this cell type (Fig. 9B). Future studies are planned to determine the function of these cells in the fungal asthma model.

3 Discussion

RANTES/CCL5 has been implicated in the host's response to asthma and allergy. In this study, we examined the therapeutic potential of directly inhibiting the pulmonary actions of this CC chemokine during *Aspergillus*-induced allergic airway disease. RANTES/CCL5-responsive cells were targeted by the intranasal delivery of a chimeric protein comprised of RANTES/CCL5 and *Pseudomonas* exotoxin A (RANTES-PE38). The intranasal delivery of RANTES-PE38 from the time of the conidia challenge (day 0) to day 15 prevented the development of all early features of *Aspergillus*-induced allergic airway disease (increased IgE, airway hyperresponsiveness, and peribronchial inflammation), but failed to prevent the chronic features of the disease (goblet cell metaplasia and mucus production). Flow cytometry and immunohistochemical staining to detect CCR5$^+$ cells clearly illustrated a reduction in the number of these cells after treatment with RANTES-PE38 from days 0 to 15 after conidia challenge. Unfortunately, due to the lack of appropriate reagents, the same analysis could not be performed for CCR1 and CCR3. Delaying the delivery of RANTES-PE38 until days 15 to 30 after conidia challenge had only a marginal effect on any of the symptoms of fungal asthma. The experimental results illustrate that RANTES/CCL5 is a primary instigator of *Aspergillus*-induced allergic airway disease, and as such

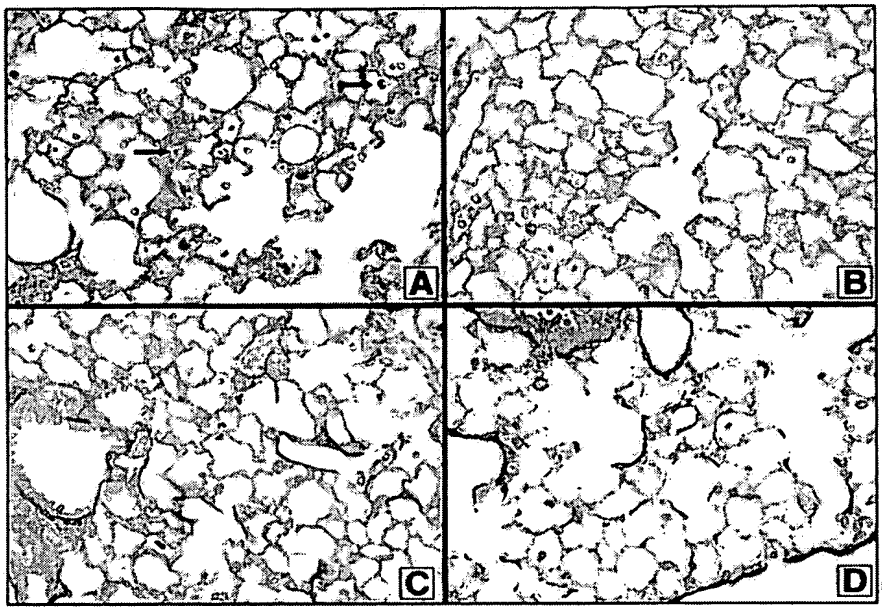

Fig. 5. GMS-stained whole-lung sections from control (A, C) and RANTES-PE38-treated (B, D) mice. In the day-0–15 group, mice received saline (A) or 1 μg of RANTES-PE38 in saline (B) intranasally 2 h before conidia challenge and every 48 h for 15 days. In the day-15–30 group, each mouse received saline (C) or 1 μg of RANTES-PE38 (D) in saline intranasally every 48 h from day 15 until day 30 after conidia challenge. Fungal material appears as black dots (arrows); the black material around the alveolar spaces is due to polysaccharide in the matrix deposition being stained by the GMS. Original magnification was 200×.

it is an appropriate target in the early treatment of this disease.

The effect of RANTES-PE38 on circulating levels of total IgE is consistent with previous studies showing the enhanced production of IgE and IgG4 by RANTES/CCL5- and MIP-1α/CCL3-stimulated B cells without affecting production of other isotypes [16]. Our findings coincide, in part, with those of Gonzalo et al. [24], who noted that the systemic administration of met-RANTES, a RANTES/CCL5 receptor antagonist [32, 33], significantly reduced peribronchial inflammation in an acute OVA model of allergic airway disease. While there was more interstitial inflammation observed in the mice treated with RANTES-PE38 than in the control mice, probably due to recruitment of cells into the lungs by the immunotoxin, peribronchial inflammation was effectively eliminated. Further, it is clear that a combination of signaling events is needed for the effective recruitment of immune cells. Macrophages and eosinophils were recruited in greater numbers in the RANTES-PE38-treated lungs, but at the appropriate time. T cells, which also express CCR5 but are usually observed much later in the model, where not recruited. The spleens of the treated mice were approximately 60% smaller than those from control animals. When splenocytes were assessed by flow cytometry, RANTES-PE38-treated spleens had fewer cells in every population examined (data not shown), again suggesting that the immunotoxin can both recruit and kill sensitive cells. Eosinophils' contribution to allergic airway hyperresponsiveness remains controversial, yet it is evident that the increased number of eosinophils in the BAL fluid of the RANTES-PE38-treated mice did not increase immunopathology.

We have previously reported transient *Aspergillus*-induced allergic airway disease in $CCR5^{-/-}$ mice [29]. This temporal airway disease was dependent on systemic RANTES/CCL5, ostensibly acting through its other receptors, CCR1 and CCR3. The $CCR5^{-/-}$ mice also exhibited reduced airway remodeling (goblet cell metaplasia, mucus hypersecretion, and fibrosis) when compared to controls. In this model, the RANTES-PE38 treatment did not reverse or attenuate typical goblet cell metaplasia. This observation was unexpected as it contrasts with our findings in the CCR5-knockout mouse [29]. However, this phenotype reflects our studies in mice that are lacking CCR4 [31], in which *A. fumigatus*-

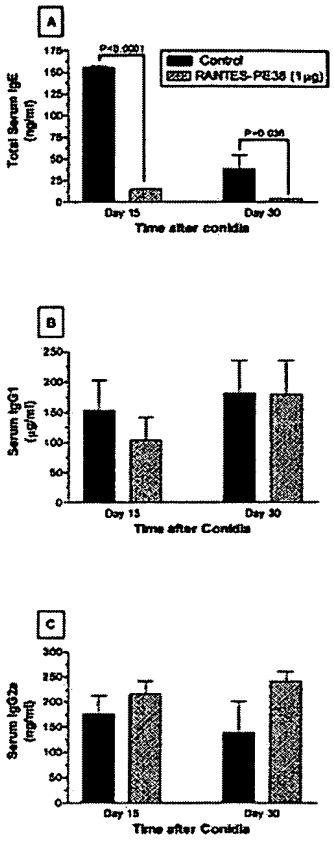

Fig. 6. Serum levels of IgE (A), IgG1 (B), and IgG2a (C) in *A. fumigatus*-sensitized mice at days 15 and 30 after live *A. fumigatus* conidia challenge. In the day-0–15 group, mice received saline or 1 μg of RANTES-PE38 in saline intranasally 2 h before conidia and every 48 h for 15 days. In the day-15–30 group, each mouse received saline or 1 μg of RANTES-PE38 in saline intranasally every 48 h from day 15 until day 30 after conidia challenge. Serum Ig levels were measured using a specific ELISA. Data are expressed as mean ± SEM; n=5–10/group/time point.

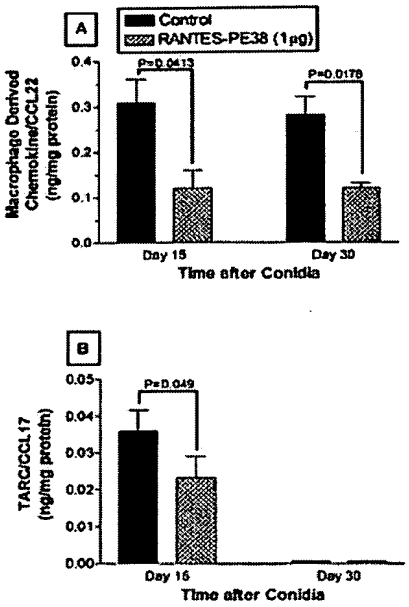

Fig. 7. Whole-lung levels of MDC/CCL22 (A) and TARC/CCL17 (B) in *A. fumigatus*-sensitized CBA/J mice at days 15 and 30 after live *A. fumigatus* conidia challenge. In the day-15 group, mice received 1 μg of RANTES-PE38 in saline or saline alone intranasally 2 h prior to conidia challenge and every 48 h for 15 days. In the day-30 group, each mouse received 1 μg of RANTES-PE38 in saline or saline alone intranasally at day 15 and every 48 h until day 30 after conidia challenge. Chemokines were measured by ELISA. Data are expressed as mean ± SEM; n=5–10/group/time point.

sensitized CCR4$^{-/-}$ mice exhibited significantly reduced airway inflammation and hyperreactivity yet exhibited prominent goblet cell metaplasia. RANTES-PE38 treatment also modulated the expression of the CCR4 ligands MDC/CCL22 and TARC/CCL17 (TARC also binds CCR8).

RANTES-PE38 treatment further mimics CCR4 deficiency [31, 34] in that F4/80$^+$ cells are drastically reduced after the introduction of *Aspergillus* conidia in both models. Immunohistochemical analysis and flow cytometry showed that RANTES-PE38 administration ablated a cell population morphologically consistent with resident lung macrophages. It seems counterintuitive that the removal of cells that are normally regarded as phagocytic could stimulate the innate immune response to increase fungal clearance. However, the heterogeneous phenotype of pulmonary macrophages has recently been the topic of much research and may illuminate the various functions of these cells.

Our working hypothesis is that CCR4$^+$ resident lung macrophages also express one or more of the RANTES/CCL5 receptors (probably CCR5) and are being removed by the immunotoxin treatment. This explains the similarity between the CCR4$^{-/-}$ phenotype and that of the RANTES-PE38-treated animals. Further, these CCR4$^+$ macrophages are responsible for the linking of the innate and acquired immune response in this model. Namely, that RANTES working through CCR5 is important in the early aspects of the disease, probably as early as the sensitization phase of the model. The co-expression of CCR5 and CCR4 on the resident macrophage popula-

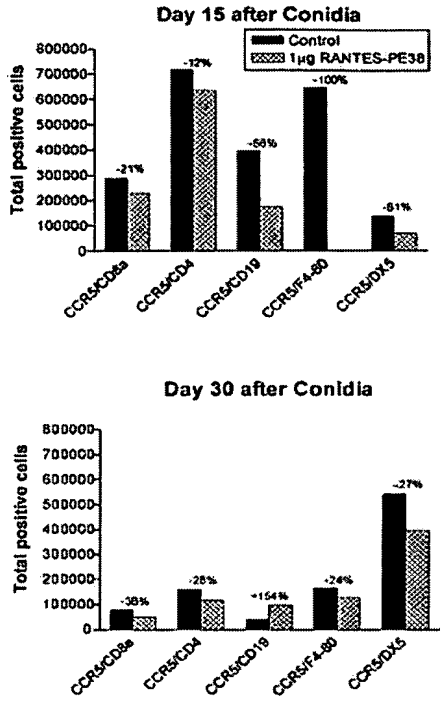

Fig. 8. Flow cytometry analysis of CCR5-positive cells from lung cell suspensions from mice treated from day 0 until day 15 (A) or from day 15 until day 30 (B) after conidia challenge. In the day-15 group, mice received 1 µg of RANTES-PE38 in saline or saline alone intranasally 2 h prior to conidia challenge and every 48 h for 15 days. In the day-30 group, each mouse received 1 µg of RANTES-PE38 in saline or saline alone intranasally at day 15 and every 48 h until day 30 after conidia challenge. Total numbers of positive cells were calculated by multiplying the positive percentage by the total number of cells retrieved from the whole lungs. Percent increase (+) or decrease (–) is reflective of the treatment group as compared with the control for each sample.

tion allows the switch from the early inflammatory phase, delineated by Th1-associated inflammation, to the Th2-dominated chronic disease. Experiments are underway to assign the relative contribution of these macrophages in the context of fungal allergy and how the interplay of these cells with other cells may be manipulated to reduce airway hyperresponsiveness.

In summary, this study illustrated a direct contribution by RANTES/CCL5 to the development of chronic *Aspergillus*-induced allergic airway disease. RANTES-PE38 appeared to modulate features of the disease, in whole or in part, through the direct targeting of CCR5-expressing cells or RANTES/CCL5-responsive cells that regulate the recruitment of CCR5-expressing cells into conidia-challenged lungs. These data reveal the major role that RANTES/CCL5-responsive cells exert in the lung during *Aspergillus*-induced disease and highlight the benefit that may be derived from targeting these cells during clinical allergy or atopic asthma.

4 Materials and methods

4.1 Chronic *Aspergillus*-induced allergic airway disease in mice

Specific pathogen-free, female CBA/J mice (Jackson Laboratories, Bar Harbor, ME) were housed in the University Laboratory Animal Medicine (ULAM) facility at the University of Michigan Medical School. Prior approval for mouse usage was obtained from ULAM. Mice were sensitized to a commercially available preparation of soluble *A. fumigatus* antigens as previously described in detail [30]. *A. fumigatus*-sensitized mice received 5×10^6 *A. fumigatus* conidia suspended in 30 µl of PBS containing 0.1% Tween-80 via an intratracheal inoculation [30].

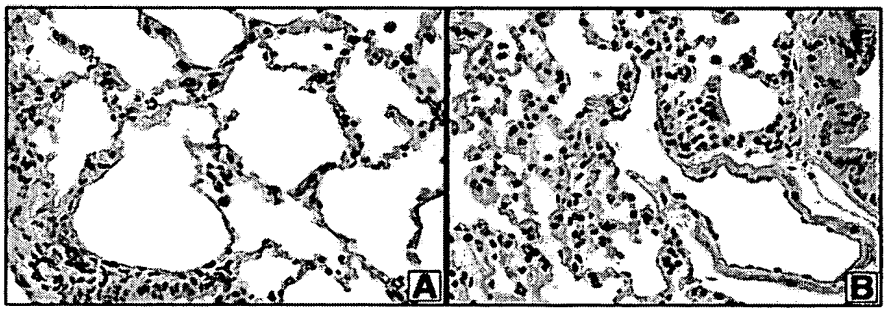

Fig. 9. Immunohistochemistry highlighting $CCR5^+$ cells in the interstitium of saline-treated (A) and RANTES-PE38-treated (B) mice. Animals were treated 2 h before conidia challenge and every 48 h thereafter until day 15. CCR5 staining appears red and counterstaining is blue. Original magnification ×200.

4.2 *In vivo* depletion of RANTES/CCL5-responsive cells in the lung with a chemokine toxin

In the present study, a fusion protein of the chemokine RANTES/CCL5 and a truncated version of *Pseudomonas* exotoxin A (RANTES-PE38) was used to target cells that express RANTES/CCL5 receptors, especially the primary RANTES/CCL5 receptor CCR5 [35]. The construction of RANTES-PE38 is described in detail elsewhere [25]. Previous studies have shown that RANTES-PE38 targets and kills CCR5-positive CHO cells but has no toxic or killing effect towards CHO expressing CXCR4 or CHO cells lacking any chemokine receptor *in vitro* [25].

As RANTES-PE38 had not been previously used *in vivo*, we selected the intranasal route of administration (the route employed in our studies with an IL-13 and *Pseudomonas* exotoxin A chimera [36]). The initial study showed that a dose of 1,000 ng, but not 200 ng, of RANTES-PE38 given intranasally had a significant inhibitory effect on the airway hyperreactivity associated with *Aspergillus*-induced allergic airway disease (not shown). The higher dose was used for subsequent studies. Groups of ten *A. fumigatus*-sensitized mice received RANTES-PE38 at the time of the conidia challenge and every 48 h until day 15 after conidia challenge. Other groups of five *A. fumigatus*-sensitized mice received RANTES-PE38 intranasally at day 15 and every 48 h until 30 days after conidia challenge. The control groups (n=5–10/group) received saline (vehicle) according to the same protocols. Airway hyperresponsiveness, inflammation, and remodeling were examined on either day 15 or 30 after conidia challenge.

4.3 Measurement of bronchial hyperresponsiveness

Bronchial hyperresponsiveness in sodium pentobarbital (0.04 mg/g of body weight; Butler Co., Columbus, OH)-anesthetized and mechanically ventilated (Harvard Apparatus, Reno, NV) mice was assessed using a Buxco plethysmograph (Buxco, Troy, NY) as previously described in detail [30]. After a baseline period of 5 min, the mice received 420 μg/kg of methacholine by tail vein injection. Airway hyperresponsiveness was calculated as previously described in detail [30]. After the assessment of airway hyperresponsiveness, mice were euthanized and a BAL was performed using 1 ml of normal saline. A 500-μl aliquot of blood was removed from each mouse. BAL fluids were centrifuged at 4,000×g for 5 min, and the pelleted cells were used for Cytospin (Shandon Scientific, Runcorn, GB; see below). Serum was obtained from each blood sample after centrifugation at 16,000×g for 10 min. Finally, whole lungs were dissected from each mouse and snap-frozen in liquid N_2 or prepared for histological analysis (see below).

4.4 Morphometric analysis of leukocyte accumulation in BAL fluid samples

Neutrophils, macrophages, eosinophils, and lymphocytes were quantified in BAL fluid samples cytospun onto microscope slides. A total of 1×10^5 BAL fluid cells were cytospun onto each slide to compensate for differences in cell retrieval. Each slide was stained with a Wright-Giemsa differential stain, and the average number of each cell type per high-powered field was determined after counting a total of 15 high-powered fields (1,000×) per slide.

4.5 Chemokine ELISA analysis

Mouse RANTES/CCL5, eotaxin/CCL11, MDC/CCL22, TARC/CCL17, and MCP-1/CCL2 levels were determined in 50-μl aliquots of whole-lung homogenates using a standardized sandwich ELISA technique [37]. Levels of each cytokine were consistently below 50 pg/ml, allowing for an accurate calculation within the linear range of each assay. Each ELISA was screened to ensure antibody specificity. The cytokine levels in each sample were normalized to total protein levels by Bradford assay.

4.6 Whole-lung histological analysis

Whole lungs from *A. fumigatus*-sensitized mice at various times after *A. fumigatus* conidia challenge were fully inflated with 10% buffered formalin, dissected, and placed in fresh formalin for 24 h. Routine histological techniques were used to paraffin-embed the entire lung, and 5-μm sections of whole lung were stained with hematoxylin/eosin (H/E), PAS, or GMS stain. Perivascular and peribronchial inflammation and structural alterations were examined using light microscopy (200×).

4.7 Flow cytometry analysis of lung cells

The lungs of RANTES-PE38-treated and control mice were aseptically removed at day 15 or 30 after conidia challenge. Lung tissues were pooled, macerated, shaken in 0.2% (wt/vol) collagenase (type IV) in RPMI with 10% fetal bovine serum for 45 min at 37°C, and washed in a balanced salt solution to remove aggregates. Cell suspensions were treated with ammonium chloride to lyse red blood cells. The resulting cell suspensions were washed, fixed in 2% paraformaldehyde for 30 min, and counted with the aid of a hemacytometer. Five-hundred thousand cells were aliquoted to clean tubes for staining. Fc binding was blocked via a 10-min incubation with anti-mouse CD16/CD32 (BD PharMingen, San Diego, CA).

The cells were stained first with anti-mouse CCR5 and then with anti-mouse CD4, anti-mouse CD8a, anti-mouse CD19, anti-mouse F4/80, or anti-mouse DX5 (all BD PharMingen)

for 30 min at 4°C in PBS supplemented with 5% fetal calf serum. Residual antibody was removed by washing between and after the antibody additions. Isotype controls for each combination of antibodies were prepared in a similar manner. Each cell population was analyzed by flow cytometry (EPICS XL; Beckman Coulter, Miami, FL). The isotype control was used to gate the population of interest and as the negative control. The total population of each cell type was corrected against the control, and total cell counts were calculated.

4.8 Immunohistochemical analysis of lung tissue sections

Formalin-fixed, paraffin-embedded histological sections were used for immunohistochemical analysis of $CCR5^+$ cells. Sections were deparaffinized and rehydrated through a xylene:alcohol series to a final wash in PBS. The slides were microwaved in a pressure cooker with 10 mM citric acid (pH 6.0) for 20 min for antigen retrieval. The sections were stained per manufacturer's instructions using the HRP-AEC Cell and Tissue Staining Kit (R&D Systems, Minneapolis, MN). A primary rat anti-mouse CCR5 mAb [25] was used at a concentration of 1:5, and a rabbit anti-rat biotinylated secondary antibody (InnoGennex, San Ramon, CA) was used at 1:20. A null control (no primary antibody) was run on each specimen to evaluate endogenous peroxidase staining. Specimens were examined using light microscopy (200×).

4.9 Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). An unpaired *t*-test was used to determine statistical significance between control and treatment groups at various times after the conidia challenge; $p<0.05$ was considered statistically significant.

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Correspondence: Jane M. Schuh, Department of Pathology, University of Michigan Medical School, Rm. 5214 Med. Sci. I, 1301 Catherine Rd., Ann Arbor, MI 48109–0602, USA
Fax: +1-734-764-2397
e-mail: janeschu@umich.edu